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Smura, Teemu

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Aseptic meningitis outbreak associated with echovirus 4 in Northern Europe in 2013–2014

Teemu Smura[a], Soile Blomqvist[b], Pekka Kolehmainen[c], Isabelle Schuffenecker[d], Bruno Lina[d], Sindy Böttcher[e], Sabine Diedrich[e], Arthur Löve[f], Mia Brytting[g], Elenor Hauzenberger[g], Susanne Dudman[h], Olga Ivanova[i,j], Alexander Lukasev[i,j], Thea Kølsen Fischer[k], Sofie Midgley[k], Petri Susi[l], Carita Savolainen-Kopra[b], Maija Lappalainen[a], Anne J. Jääskeläinen[a,*]

[a] University of Helsinki and Helsinki University Hospital, Virology and Immunology, Helsinki, Finland
[b] National Institute for Health and Welfare (THL), Department of Health Security, Helsinki, Finland
[c] University of Helsinki, Department of Virology, Helsinki, Finland
[d] Institut des Agents infectieux des HCL, CNR des enterovirus, Hôpital de la Croix-Rousse & Université de Lyon, CIRI INSERM U1111, UCBL, Lyon, France
[e] Robert Koch Institute, Berlin, Germany
[f] Landspitali, National University Hospital, Reykjavik, Iceland
[g] Folkhälsomyndigheten, Public Health Agency of Sweden, Solna, Sweden
[h] Norwegian Institute of Public Health, Oslo, Norway
[i] Chumakov Federal Scientific Center for Research and Development, Moscow, Russia
[j] Sechenov First Moscow State Medical University, Moscow, Russian Federation
[k] Statens Serum Institut, Copenhagen, Denmark
[l] University of Turku, Institute of Biomedicine, Turku, Finland

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ABSTRACT

Picornaviruses (family Picornaviridae) are small, nonenveloped, positive-sense, single-stranded RNA viruses. The members of this family are currently classified into 47 genera and 110 species. Of picornaviruses, entero- and parechoviruses are associated with aseptic meningitis. They are transmitted via fecal-oral and respiratory routes, and occasionally, these viruses may cause a brief viremia and gain access to central nervous system (CNS). During the diagnostic screening of entero- and parechovirus types in Finland in year 2013 − 14, we detected a cluster of echovirus 4 (E4) infections in young adults and adolescents. As E4 is infrequently detected in Finland, we contacted several Northern and Central European laboratories that conduct routine surveillance for enteroviruses and, for those who have had E4 cases, we send a query for E4 sequences and data. Here we report CNS infections caused by E4 in Finland, Sweden, Norway, Denmark, Iceland and Germany in 2013 and 2014, and show that the E4 detected in these countries form a single lineage. In contrast, E4 strains circulating in these countries preceding the year 2013, and those circulating elsewhere in Europe during 2013–2014, formed several independent clusters.

1. Introduction

Picornaviruses (family *Picornaviridae*) are small, non-enveloped, plus-sense, single-stranded RNA viruses. The members in this family are currently classified into 40 genera and 94 species (www.picornaviridae.com) and include several notorious pathogens including entero- and parechoviruses, which are the most common causes of viral (aseptic) meningitis [1,2]. Both entero- and parechoviruses are transmitted via fecal-oral and respiratory routes. Occasionally, these viruses cause a brief viremia and gain access to CNS. In particular, several enterovirus (genus *Enterovirus*; EV) types including many echoviruses (e.g. E9, E11 E30) and numbered enteroviruses (e.g. EV-A71) and human parechovirus (genus *Parechovirus*) types 3 and 4 (PeV-A3, PeV-A4) have been associated with central nervous system (CNS) infections causing clinical conditions such as meningitis, encephalitis and acute flaccid paralysis (AFP) [3–6,40,41]. Entero- and parechovirus infections are common in infants (up to 2-years of age) and enterovirus infections in older children and adolescents. In temperate zones, the infections have seasonal pattern with incidence peak from late summer to late autumn. Typically, several different enterovirus types circulate during each year

* Corresponding author at: University of Helsinki and Helsinki University Hospital, Virology and Immunology, Topeliuksenkatu 32, 00029 HUS, Helsinki, Finland.
E-mail address: anne.jaaskelainen@helsinki.fi (A.J. Jääskeläinen).

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[3,6,7].

During a routine surveillance, we detected a cluster of echovirus 4 (E4) infections in Finland, especially in adolescents (up to 15-years of age) and adults (up to 31-years of age) with reported central nervous system infection. Since E4 is infrequently detected in Finland [8], and to gain more detailed insight into the molecular epidemiology of E4, a query regarding E4 prevalence was sent to several Northern and Central European laboratories that conduct routine surveillance for enteroviruses. Here we report the clinical aspects of E4 neurological infection identified in Finland and the molecular-characteristics of E4 circulated in Finland, Sweden, Norway, Denmark, Iceland and Germany. In contrast, E4 strains circulating in these countries preceding the year 2013, and those circulating elsewhere in Europe formed several independent clusters. In addition to E4 findings, other EV and PeV-A cases during 2013–2014 in Finland are reported in this study.

2. Materials and methods

2.1. Sample collection in Finland

Altogether, 594 and 678 routine EV/PeV-A diagnostic samples collected in 2013 and 2014, respectively, in Helsinki/Uusimaa hospital district located in the Southern Finland, were included in the study. The samples comprised cerebrospinal fluid (CSF), serum, feces, skin lesion/vesicle and throat swabs sent for EV/PeV nucleic acid detection test (NAT, described below) or for virus isolation (VC; described in [5]) at Helsinki University Hospital (HUSLAB), Finland. From individuals, different samples types with different combinations were sampled during the year.

2.2. Virus isolation in cell cultures

Cell lines of Vero, GMK and A549 were routinely used for all samples types. The culture media consisted of 2% fetal bovine serum (FBS) and antibiotics (penicillin streptomycin) and nystatin, and cell bottles were incubated at 37 °C, and microscoped daily-base for cytopathic effect (CPE).

2.3. RT-qPCR and genotyping assays

RNA was extracted from biological samples and cell-cultured positive supernatant using either QIAamp Viral RNA kit (Qiaqen Inc., Valencia, CA, USA) or EasyMag (bioMerieux) according to the manufacturer's instructions. The extracted RNA was either processed immediately or frozen at −70 °C and stored for further analysis.

The samples were tested for EV, PeV-A and human rhinovirus nucleic acids by an in-house real time multiplex RT-qPCR for EV and PeV-A, a real time RT-qPCR for human rhinoviruses, and a commercial rapid molecular Xpert® EV test (Cepheid) for CSF samples. The validated in-house real time multiplex RT-qPCR for EV and PeV-A was carried out using the primers of Par30 (400 nM, 5′-GGTACCTTCTGGGCATCC TTC-3′; [9]) and Par31 (400 nM, 5′-CTGGGGCCAAAAGCCA-3′ [10]), PeV-A-probe (200 nM, 6′FAM-5′-AAACACTAGTTGTAHGGCCC-3′-MGB [11]), EV-primers (400nnM, 5′-ACATGGTGTGAAGAGTCTATTGA GCT-3′; 400 nM, 5′-CCAAAGTAGTCGGTTCCGC-3′) and probe (50 nM, VIC-5′-TCCGGCCCCTGAATGCGGCTAAT- 3′-MGB) described by Dierssen et al. [12], and Superscript® III Platinum® One-step qRT-PCR System (Invitrogen, Carlsbad, CA, USA).

The validated real time RT-qPCR for human rhinoviruses was carried out using in-house primers (500 nM, 5′- AAGGTGTGAAGAGCCCC GTGT- 3′; 750 nM, 5′-GAAACACGGACACCCAAAGTAGT- 3′) and probe (250 nM, 6′FAM - 5′-CCGGCCCCTGAATGYGGCTAACCT-3′-MGB) in addition to Superscript® III Platinum® One-step qRT-PCR System (Invitrogen, Carlsbad, CA, USA). Both multiplex-EV-PEV-A and human rhinovirus real time RT-qPCRs were carried out using the PCR conditions of 15 min at 50 °C, 2 min at 95 °C, followed by 45 cycles of 15 s at 95 °C and 50 s at 60 °C. The Xpert® EV test (Cepheid) was carried out according to manufacturer´s instructions.

In order to type the virus strains, samples that were EV or PeV-A RNA positive, or showed picornavirus-like cytopathic effect (CPE) in virus cultivation, were subjected to conventional RT-PCRs targeting the VP1 coding region (Leitch et al. 2011). For EV and PeV-A, method and primers (each in final concentration of 500 nM) modified from Leitch et al. [13] with One-step RT-PCR kit (Invitrogen) for the first round RT-PCR and Phusion Flash (Thermo Scientific) for the second round PCR, and primers (each in final concentration of 500 nM) and PeV-RT-PCR method described in Kolehmainen et al. [11], were used, respectively. Rhinovirus positive samples were not typed. The PCR products (approx.700 − 900bp) were sequenced at DNA sequencing and Genomic service (Helsinki University, Institute of Biotechnology, Viikki, Helsinki). Feces, CSF, and lesion/vesicle swabs sent for virus isolation using A549, GMK and Vero cells and CPE positives were screened with real time multiplex RT-qPCR for EV and PeV-A, and further VP1-typed, accordingly, using the methods described above.

2.4. Sequence data collection

A query to obtain VP1 sequences from E4 samples detected, either during 2013 − 14 or prior to that, was sent to a few European laboratories that conduct routine surveillance for enteroviruses. Altogether seven laboratories, including The Public Health Agency of Sweden, Norwegian Institute of Public Health (Norway), Statens Serum Institut in Denmark, National University Hospital in Iceland, National Reference Lab for Poliomyelitis and Enteroviruses in Germany, M.P. Chumakov Institute of Poliomyelitis & Viral Encephalitides in Russia and University Hospital of Lyon, France, reported E-4 infections, whereas Österreichische Agentur für Gesundheit und Ernährungssicherheit in Austria and University of Edinburgh in UK Scotland had no confirmed E4 cases.

In addition to strains sequenced in this study, all available E4 VP1 sequences were downloaded from the GenBank. The sequences were aligned using ClustalW algorithm implemented in MEGA7 software [14]. The best fit substitution model was estimated using jModeltest 2.1.10 [15]. Phylogenetic tree was constructed using Bayesian Monte Carlo Markov Chain (MCMC) method implemented in BEAST version 1.10 [16] using two different clock models (strict clock and log normal relaxed clock) and three demographic models (constant, Bayesian skyline and exponential models) [17,18]. The marginal likelihoods of different model combinations were estimated using path sampling and stepping stone methods [19] and Bayes factors (BF) were calculated for each pair of models. The final analysis was conducted using K2 + G model of substitution, lognormal relaxed clock and Bayesian skyline demographic model, which showed logBF > 80 to the other model combinations in both stepping stone and path sampling analyses.

3. Results

In Finland, altogether 30 routine diagnostic samples (30/594 samples, 5.1 %; 19 NAT and 11 VC; three individuals had both NAT and VC positive) from 27 patients in 2013, and 46 samples (46/678 samples, 6.8 %; 40 NAT and 6 VC; one individual had both NAT and VC positive) from 45 patients in 2014, were positive for picornaviruses.

Sequencing was conducted for 45 samples acquired from 41 individuals (median age 6, range 0 − 59-years; 21 males, 20 females; year 2013, 15 individuals, year 2014, 26 individuals; Table 1) out of the 72 individuals (year 2013, 27 individuals; year 2014, 45 individuals), in total. Most common sample types were CSF (N = 15; 15/45 33 %), vesicle (N = 12; 12/45, 27 %) and feces (N = 11; 11/45, 24 %), followed by serum (N = 4; 4/45, 9%) and throat swab (N = 3; 3/45, 7%). Samples originated mostly from hospital district of Helsinki and Uusimaa.

Coxsackievirus A6 (CVA6) was the most common finding [N = 12;

Table 1
Forty-one entero- and parechovirus findings, which were sequenced during the years 2013-2014 in Helsinki University Hospital Laboratory (HUSLAB, Helsinki, Finland).

Patient	Age (y/m)	Gender (f/m)	Hospital District	Sample type	Sampling time (year/month)	Sequenced virus type
1 – 13	5y	f	Southwest Finland	throat swab	2013/07	CVA4
2 – 13	48y	f	North Savo	CSF	2013/07	E2
3 – 13	1 m	f	Helsinki and Uusimaa	CSF	2013/08	E11
				feces	2013/08	E11
4 – 13	23y	f	Helsinki and Uusimaa	CSF	2013/09	E30
				feces	2013/09	E30
5 – 13	1 m	f	Helsinki and Uusimaa	CSF	2013/09	CVB4
6 – 13	14y	m	Helsinki and Uusimaa	feces	2013/09	CVB4
7 – 13	2w	f	Helsinki and Uusimaa	feces	2013/10	CVA16
8 – 13	13y	f	Helsinki and Uusimaa	feces	2013/10	CVB3
9 – 13	2y7m	f	North Savo	vesicle	2013/10	CVA6
10 – 13	1 m	m	North Savo	serum	2013/10	PEV-A3
11 – 13	4d	m	Helsinki and Uusimaa	throat swab	2013/10	CVA16
12 – 13	1 m	f	Helsinki and Uusimaa	feces	2013/11	E25
13 – 13	1 m	m	Helsinki and Uusimaa	feces	2013/11	CVB1
				CSF	2013/11	CVB1
14 – 13	12y	m	Helsinki and Uusimaa	vesicle	2013/11	CVB3
15 – 13	19y	m	Helsinki and Uusimaa	vesicle	2013/12	CVA6
1 – 14	7m	m	Helsinki and Uusimaa	vesicle	2014/01	CVA2
2 – 14	32y	m	Helsinki and Uusimaa	vesicle	2014/02	CVA6
3 – 14	25y	f	Helsinki and Uusimaa	CSF	2014/05	E4
4 – 14	14y	m	Helsinki and Uusimaa	feces	2014/05	E4
5 – 14	12y	m	Central Finland	CSF	2014/06	E4
6 – 14	24y	f	Helsinki and Uusimaa	CSF	2014/07	E4
7 – 14	27y	f	Helsinki and Uusimaa	feces	2014/07	E4
8 – 14	1y	m	North Savo	vesicle	2014/07	CVA6
9 – 14	15y	f	Helsinki and Uusimaa	CSF	2014/09	E4
10 – 14	54y	f	Helsinki and Uusimaa	vesicle	2014/09	CVA16
11 – 14	16y	f	Helsinki and Uusimaa	CSF	2014/09	E4
12 – 14	14y	m	Helsinki and Uusimaa	CSF	2014/09	E30
13 – 14	9m	m	Helsinki and Uusimaa	vesicle	2014/09	CVA6
14 – 14	1y11 m	f	North Karelia	serum	2014/10	CVA6
15 – 14	1 m	m	North Savo	serum	2014/09	PEV-A3
16 – 14	1.5m	m	Helsinki and Uusimaa	feces	2014/10	PEV-A3
17 – 14	6y	m	Helsinki and Uusimaa	feces	2014/10	CVB4
				CSF	2014/10	CVB4
18 – 14	27y	m	North Karelia	CSF	2014/10	E4
19 – 14	59y	m	South Karelia	throat swab	2014/10	CVA6
20 – 14	3m	m	Central Finland	CSF	2014/10	CVA6
21 – 14	31y	f	Helsinki and Uusimaa	CSF	2014/11	E9
22 – 14	6m	f	Helsinki and Uusimaa	vesicle	2014/11	CVA6
23 – 14	39y	f	Central Finland	serum	2014/11	CVA6
24 – 14	5m	m	Helsinki and Uusimaa	vesicle	2014/11	CVA16
25 – 14	4y	m	Helsinki and Uusimaa	vesicle	2014/11	CVA6
26 – 14	6m	f	Southwest Finland	vesicle	2014/12	CVA6

CSF, cerebrospinal fluid; NA, not available; NPA, nasopharyngeal aspirate; m, months; y, years; E, echovirus; CVA, coxsackievirus A; CVB, coxsackievirus B.

12/41, 29 %), followed by echovirus 4 (E4, N = 8; 8/41, 20 %), CVA16 (N = 4; 4/41, 10 %), CVB4 (N = 4; 10 %), E30 (N = 3; 3/41, 7%), CVB1 (N = 2; 2/41, 5%) and CVB2 (N = 2; 5%) (Table 1). In addition, CVA2, CVA4, E2, E9 and E25 (N = 1; 1/41, 2%) were detected during the study period. CVA2, CVA6 and CVA16 were detected most commonly from vesicular lesion, whereas the most common sample type for E2, E4, E9, E11, E30, CVB1 and CVB4 was CSF (Table 1). In addition, two PeV-A3 isolates were detected from a serum sample originated from North Savo, and a fecal sample from Helsinki and Uusimaa region (Table 2). Both patients were neonates under two months old. Clinical findings of E4 and other EVs and PeV-A3 cases in hospital district of Helsinki and Uusimaa (HUS, Finland) were collected retrospectively and are summarized in Table 2. From other hospital districts in Finland, the data were not available (Table 2).

There were some differences in the number of circulating EV types between 2013 and 2014. More EV cases were detected in 2014 than during the year of 2013, however, the numbers are too low to conclude reliable statistical difference. In 2014, there was more of E4 and CVA6 infections. Since 2008, CVA6 has been detected annually as the causative agent of hand, foot and mouth disease in Finland [7,39]. Interestingly, the number of E4 detections among patients with suspected CNS infection increased during the year 2014. In total two males and five females (median age 24, range 14–31; median of 4 days in hospital, range 0–5) were diagnosed for E4 infection. The C-reactive protein levels were elevated in 6 out of seven E4 infected patients.

Since E4 is infrequently detected in Finland [8], a query for E4 sequences was sent and altogether five countries reported similar cases in 2013–2014 (Table 3); four cases were reported from Sweden, one from Norway, five from Denmark, two from Iceland and ten from Germany. In addition, 11 cases of E4 infections were reported from Germany during years 2006–2012 (Table 3). Fourteen cases were reported from Lyon, France between years 2008–2015, but none of these had occurred during 2013–2014 (Table 3). Three cases were reported from Russia during years 2003–2006, and one case from Denmark in 2010 (Table 3). In addition, six older E4 strains (years 1991–2008) from Finland were included in the analysis. The country, median age, sample types, year, age and sex of patients, and diagnosis/symptoms (when available) are listed in Table 3.

The phylogenetic analysis suggested three highly divergent major groups for E4 (Fig. 1). Of these, one contained a sole prototype strain Pesacek isolated in Connecticut, USA in the year 1951, which showed 16.2–23.2 % divergence at nucleotide level (3.2–10.9 % at amino acid

Table 2
Clinical findings of E4 and other EV-Bs and PeV-A3 cases in hospital district of Helsinki and Uusimaa.

Virus detected (Age or median age)	Sex	Age	Date of hospitalization	Days in Hospital	Clinical Symptoms	C-reactive protein (mg/l; < 5 mg/l)	Blood leucocyte count (E9/l[a,b])	Blood thrombocyte count (E9/l[a,b])	Protein amount in CSF (mg/l)	Treatment	Underlying disease/s
E4 (24y)	M	14y	13th of May, 2014	5	Very high fever (> 40 °C), headache	70 ↑	8.1[a]	175[a] ↓	NA	Ceftriaxone	Not reported
	F	25y	19th of May, 2014	4	Fever, headache	20 ↑	6.9[b]	154[b]	684[b] ↑	Ceftriaxone, doximycin, aciclovir	Asthma, gastroesophageal reflux
	F	24y	11th of July, 2014	3	Fever, headache	17 ↑	NA	NA	988[b] ↑	No antimicrobial treatment	Not reported
	F	27y	15th of July, 2014	4	Fever, infection non aliter specificatus (NAS), suspicion of pyelonephritis	116 ↑	7.4[b]	157[b]	ND	Ceftriaxone	Healthy gravida (36w + 5d)
	M	14y	8th of September, 2014	0	Fever, nausea, headache	4	10[a]	184[a] ↓	436[a]	No antimicrobial treatment	Not reported
	F	15y	17th of September, 2014	5	Fever, cough, deteriorated condition, headache	24 ↑	4.8[a]	185[a]↓	804[a] ↑	No antimicrobial treatment	Migraine
	F	31y	2nd of November, 2014	2	Nausea, vertigo, headache	9 ↑	11.2[b]	242[b]	307[b]	No antimicrobial treatment	Not reported
E11 (4wks)	F	4ws	23th of August, 2013	5	Fever, irritable	4	5.5[c]	136[c]↓	524[c]↑	G-penicillin, gentamycin	Not reported
E30 (23y)	F	23y	13th of September, 2013	4	Headache, fever, nausea, sepsis suspected	19 ↑	7.5[d]	113[d]↓	875[d]↑	Ceftriaxone, vankomycin, aciclovir	Not reported
CVB4 (6y)	M	14y	16th of September, 2013	3	Headache, impaired vision, fever	< 5	8.9[d]	234[d]	669[d]↑	Doximycin	Not reported
	F	4ws	20th of September, 2013	3	Fever	< 5	10.6[a]	293[c]	573[c]↑	Ceftriaxone, aciclovir	Not reported
	M	6y	6th of October, 2014	2	Headache, vomiting, absent	< 5	14.2[c]	334[c]	330[c]↑	aciclovir	Not reported
CVB3 (13y)	F	13y	23th of October, 2013	3	Fever, vertigo, palpitation, myocarditis suspected	< 3	8.6[d]	308[d]	ND	No antimicrobial treatment	Not reported
E25 (4wks)	F	4ws	12th of November, 2013	3	Fever, irritable	< 5	5.2[c]	401[c]	353[c]↑	G-penicillin	Not reported
CVB1 (4wks)	M	4ws	27th of November, 2013	4	Fever, irritable	< 5	4.2[c]	452[c]	5055[c]↑	Kefuroxime, aciclovir	Not reported
PeV-A3 (6wks)	M	6ws	1st of October, 2014	5	Fever, deteriorated condition	< 5	9.8[c]	321[c]	182[c]	G-penicillin, aciclovir	Not reported

EV-B, enterovirus B; PeV-A3, human parechovirus 3; NA, not available; ND, not detected; y, years; ws, weeks. ↑, over normal range. ↓, below normal range.

[a] Normal range: leucocyte count 4.5–13 E9/l ; thrombocyte count 200–450 E9/l; Protein in CSF 150–450 mg/l.

[b] Normal range: leucocyte count 3.4–8.2 E9/l; thrombocyte count: 150–360 E9/l; Protein in CSF 150–450 mg/l.

[c] Normal range: 1 m-1y leucocyte count 6–17.5 E9/l, 2y-6y 5–14 E9/l; thrombocyte count 1 m – 16y 200–450 E9/l; Protein in CSF 150–300 mg/l.

[d] Normal range: leucocyte count 13y-16y 4.5–13 E9/l, > 16y 3.4–8.2 E9/l; thrombocyte count 1 m – 16y 200–450 E9/l; > 16y 150–360 E9/l; Protein in CSF 150–450 mg/l.

Table 3
E4 infections reported by the collaborating laboratories via E4 query.

Country (median age, years)	Age (y/m)	Sex (f/m)	Region/City/Country	Diagnosis/symptoms reported	Sample type	Sampling time (year/month)	Virus
Sweden (22)	17y	f	Stockholm	meningitis	CSF	2014/04	E4
	20y	f	Stockholm	meningitis	CSF	2014/05	E4
	23y	f	Uppsala	meningitis	feces	2014/06	E4
	38y	m	Stockholm	meningitis	CSF	2014/08	E4
					feces	2014/09	E4
Norway (44)	44y	f	Kristiansand	meningitis/encephalitis	feces	2014/06	E4
Iceland (30)	24y	NA	Iceland	meningitis	CSF	2013/10	E4
	35y	NA	Iceland	meningitis	CSF	2014/08	E4
Germany (25)	25y	m	Homburg/Saar	meningitis	CSF	2013/NA	E4
	42y	m	Homburg/Saar	meningitis	feces	2013/NA	E4
	42y	m	Homburg/Saar	meningitis	feces	2013/NA	E4
	29y	f	Halle (Saale)	meningitis	CSF	2014/NA	E4
	7y	m	Potsdam	meningitis	feces	2014/NA	E4
	16y	m	Münster	meningitis	feces	2014/NA	E4
	3y	m	Oberschleißheim	meningitis	feces	2014/NA	E4
France (21)	1 m	Na	Lyon	fever	CSF	2008/09	E4
	4y	Na	Lyon	unknown	CSF	2008/05	E4
	11y	Na	Lyon	unknown	throat	2008/07	E4
	14y	Na	Lyon	unknown	NPA	2008/08	E4
	24y	m	Lyon	meningitis	CSF	2011/07	E4
	40y	f	Lyon	meningitis	CSF	2011/09	E4
	21y	f	Lyon	meningitis	CSF	2011/09	E4
	24y	f	Lyon	meningitis	CSF	2012/07	E4
	21y	f	Lyon	meningitis	CSF	2012/07	E4
	23y	m	Lyon	meningitis	CSF	2012/08	E4
	9y	f	Lyon	meningitis	CSF	2012/09	E4
	6y	m	Lyon	meningitis	CSF	2012/09	E4
	22y	m	Lyon	meningitis	CSF	2012/09	E4
	31y	m	Lyon	meningitis	CSF	2015/10	E4
Germany (11)	8y	m	Hamburg	meningitis	feces	2006/NA	E4
	15y	m	Stuttgart	meningitis	unknown	2006/NA	E4
	10y	f	Münster	meningitis	feces	2007/NA	E4
	4y	m	Bocholt	meningitis	feces	2007/NA	E4
	5y	f	Münster	meningitis	CSF	2007/NA	E4
	7y	m	Frankfurt	unknown	feces	2008/NA	E4
	11y	m	Frankfurt	meningitis	feces	2009/NA	E4
	15y	m	Würzburg	meningitis	feces	2011/NA	E4
	12y	m	Neumünster	meningitis	feces	2012/NA	E4
	14y	m	Neumünster	meningitis	CSF	2012/NA	E4
	15y	f	Neumünster	meningitis	feces	2012/NA	E4
Russia (11)	11y	m	Kyrgyzstan	acute infection	NA	2005/NA	E4
	3y	f	Russia	meningitis	NA	2006/NA	E4
	17y	f	Russia	meningitis	NA	2006/NA	E4

F, female; m, male; CSF, cerebrospinal fluid; NA, not available; NPA, nasopharyngeal aspirate; m, months; y, years; E, echovirus.

level) to the other clusters, whereas the two strains from Poland (year 2001) showed 17.8–23.9 % divergence at nucleotide level (5.2 − 13.8 % at amino acid level) to the other clusters. All the other strains (n = 123) formed a large cluster with high posterior probability support and a maximum of 22.3 % intra-cluster divergence at nucleotide level (12.2 % at amino acid level). This large cluster was further divided into several sub-clusters.

The E4 strains sequenced in this study grouped into several distinct sub-clusters (Fig. 1). Notably, there was both geographical and temporal variation in the clustering pattern. The 'epidemic' strains (2013–2014) from Germany, Denmark, Sweden, Finland, Norway and Iceland clustered together. Three strains from Neumunster, Germany (2012) formed an outgroup in this cluster. These two clusters rooted to a strain isolated in Hamburg, Germany in the year 2006 (Table 3). All of these strains clustered with E4 strains detected in Kenya in the year 1999.

The strains from Germany (2003–2009) and Lyon, France (2008–2015) (Table 3) formed another large cluster together with the strains previously sequenced in Tunisia [20]. The other strains sequenced in this study formed smaller clusters interspersed throughout the phylogenetic tree, often with no clear geographic pattern. Altogether, this suggests efficient geographical spread of E4 lineages.

4. Discussion

Echovirus 4 (E4) belongs to *Enterovirus B* species within family *Picornaviridae*, and has previously caused outbreaks of viral meningitis in several countries, including United Kingdom (Scotland), Spain Israel, Italy, Greece, Australia and Tunisia [21–29]. Long-term surveillance data suggests that E4 outbreaks are followed by irregular intervals of low activity [30]. In addition to E4, meningitis cases caused by other echoviruses have been reported regularly in Europe and Asia [31–36].

Here, we report clusters of E4 strains in Finland, Sweden, Norway, Denmark, Iceland and Germany, during the years 2013–2014. Clustering of the 'epidemic' strains to E4 strain 06–40 from Hamburg, Germany in 2006, and E4 strains detected in Kenya in 1999 indicated common origin. However, due to the relatively high genetic distance between the Kenyan strains and the strain 06–40 from Germany (estimated tMRCA = 1987 [95 % HPD 1974–1997]), the transmission pathway to Germany cannot be inferred based on the current data. Most likely, this E4 lineage has circulated silently for a prolonged period.

Notably, none of the strains isolated in Lyon, southern France (years 2008 − 15, mainly 2012) grouped with the epidemic strains detected in this study, but instead formed two distinct clusters with strains isolated mainly in Europe). Thus, the increased E4 circulation in France, on one side, and in Germany and Northern Europe, on the other side, occurred almost in parallel (2012–2014), but was caused by distinct E4 lineages

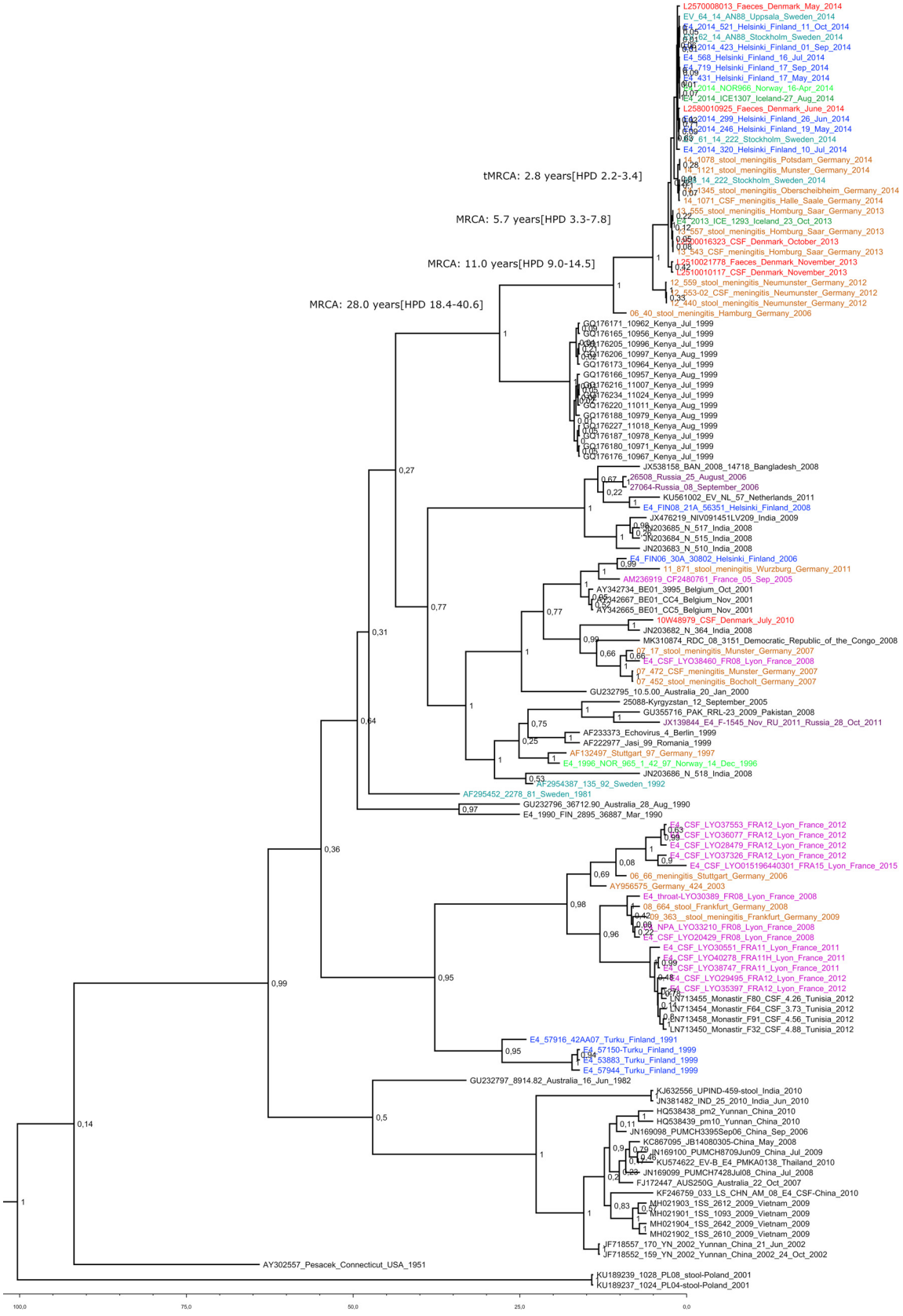

Fig. 1. Phylogenetic tree of VP1 sequences of E4 available in GenBank (NCBI) and acquired via query carried out in this study.

and was not epidemiologically related. This observation is concordant with the hypothesis of herd immunity fluctuations driving multi-year patterns of EV circulation [37].

The strains isolated in Germany, Denmark, Finland, Sweden, Norway and Iceland before the year 2012 (with the exception of the strain 06–40 from Hamburg, year 2006), did not cluster with the

epidemic E4 strains occurring during the years of 2012–2014. Most notably, the lineages that were found in Finland in 1999 and 2008 were not linked to the 2012–2014 E4 incidence surge, and apparently became extinct in Europe. The emergence-expansion-extinction of E4 subclusters was similar to the circulation patterns observed for E30 in Europe and in Russia [38,39].

In the Finnish patient cohort, the clinical findings of the patients were available from Helsinki and Uusimaa district. The E4 infected patients were adolescents or young adults [5 females, 2 males; median age: 24y [14–31], and showed symptoms for CNS infection. All but one patient had slightly elevated C-reactive protein levels. The patient with normal C-reactive protein level was not admitted to hospital, while the other E4 cases were hospitalized from two to five days. Six out of eight E4 strains were isolated/detected from CSF (1 patient in Jyväskylä, and 5 in Helsinki and Uusimaa districts), during the study period. Interestingly, two E4 cases occurred in May but rest of the E4 cases were detected in timeframe of July until November 2014, which is a more typical time of the year for season of EV infections in Finland. In 2013, coxsackievirus A infections were less abundant than in 2014 suggesting a small CVA6 epidemic in Finland as well during that year.

Overall, E4 virus outbreak was detected in several northern European countries with severe clinical outcome in 2013 − 14. This together with the low prevalence of E4 associated disease incidence in the previous years in several countries suggest that there was an immunologically naïve population that was susceptible to severe E4 infections and resulting in the circulation of epidemic strains in Germany, Finland, Sweden, Denmark, Norway and Iceland.

CRediT authorship contribution statement

Teemu Smura: Formal analysis, Data curation, Methodology, Validation, Writing - original draft, Writing - review & editing. **Soile Blomqvist:** Investigation, Formal analysis, Validation, Writing - original draft, Writing - review & editing. **Pekka Kolehmainen:** Methodology, Writing - review & editing. **Isabelle Schuffenecker:** Investigation, Data curation, Writing - review & editing. **Bruno Lina:** Investigation, Data curation, Writing - review & editing. **Sindy Böttcher:** Investigation, Data curation, Writing - review & editing. **Sabine Diedrich:** Investigation, Data curation, Writing - review & editing. **Arthur Löve:** Investigation, Data curation, Writing - review & editing. **Mia Brytting:** Investigation, Data curation, Writing - review & editing. **Elenor Hauzenberger:** Investigation, Data curation, Writing - review & editing. **Susanne Dudman:** Investigation, Data curation, Writing - review & editing. **Olga Ivanova:** Investigation, Data curation, Writing - review & editing. **Alexander Lukasev:** Investigation, Data curation, Writing - review & editing. **Thea Kølsen Fischer:** Investigation, Data curation, Writing - review & editing. **Sofie Midgley:** Investigation, Data curation, Writing - review & editing. **Petri Susi:** Investigation, Data curation, Writing - review & editing. **Carita Savolainen-Kopra:** Investigation, Data curation, Writing - review & editing. **Maija Lappalainen:** Investigation, Data curation, Writing - review & editing. **Anne J. Jääskeläinen:** Data curation, Methodology, Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision.

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